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TITLE: The Role of U2AF1 Mutations in the Pathogenesis of Myelodysplastic Syndromes

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
U2AF1 mutations occur in up to 11% of myelodysplastic syndrome (MDS) patients. To study the effects of the most common *U2AF1* mutation, U2AF1(S34F), on hematopoiesis and pre-mRNA splicing *in vivo*, we created doxycycline-inducible U2AF1(WT) and U2AF1(S34F) transgenic mice. Following transgene induction, U2AF1(S34F) mice have reduced WBCs, increased hematopoietic stem/progenitor cells, and increased HSC cell cycling compared to U2AF1(WT) mice. U2AF1(S34F) stem cells are at a competitive disadvantage compared to control cells, suggesting that the increase in HSC cell cycling following U2AF1(S34F) expression may lead to stem cell exhaustion. Next, we compared RNA splicing in progenitor cells from U2AF1(S34F) and U2AF1(WT) mice using whole transcriptome RNA-seq. We identified 460 splicing junctions that were differentially expressed in U2AF1(S34F) samples compared to U2AF1(WT). We validated several homologous dysregulated junctions (i.e., across species) in MDS patient bone marrow samples that have mutant U2AF1(S34F) versus U2AF1(WT). Together, these results suggest that mutant U2AF1 expression contributes to the altered hematopoiesis and pre-mRNA splicing observed in patients with *U2AF1* mutations. This study also identifies changes in gene isoform expression unique to *U2AF1* mutations that may have functional significance for MDS pathogenesis, which is being investigated in ongoing studies.

15. SUBJECT TERMS-
Myelodysplastic Syndromes, Splicing, Spliceosome, Mouse Model, Hematopoiesis, RNA-seq, U2AF1

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1. INTRODUCTION:

The goal of this project is to understand the mechanism of disease pathogenesis induced by *U2AF1* mutations in myelodysplastic syndromes (MDS). U2AF1 is a key spliceosome protein that binds the AG dinucleotide of the 3' splice acceptor site in pre-mRNA introns during splicing and is mutated in up to 11% of MDS patients, making it one of the most commonly mutated genes in MDS. Overall, mutations in spliceosome genes occur in up to ~50% of patients with MDS, further implicating altered pre-mRNA splicing in disease pathogenesis. We hypothesize that *U2AF1* mutations result in altered mRNA splicing in hematopoietic cells, and thereby lead to altered progenitor/stem cell function and ineffective hematopoiesis. In this project, we will test our hypothesis in the following Specific Aims. <u>Specific Aim 1. We will determine whether the U2AF1(S34F) mutation alters hematopoiesis in vivo</u>. We will inducibly express wild-type and S34F mutant (resulting from the most common *U2AF1* mutation) human *U2AF1* cDNAs in mice and determine the contribution of mutant U2AF1 to MDS pathogenesis by comprehensively evaluating basal hematopoiesis and stem cell function. <u>Specific Aim 2. We will use RNA-Seq to identify alternatively spliced genes in primary hematopoietic progenitor cells harvested from U2AF1(S34F) mutant mice</u>. We will identify alternatively spliced genes induced by *U2AF1* mutations by performing transcriptome sequencing (RNA-Seq) using RNA isolated from wild-type and mutant bone marrow progenitors. Candidate genes with alternative splicing will be interrogated in MDS patient samples with and without *U2AF1* mutations.

2. KEYWORDS:

Myelodysplastic Syndromes
Splicing
Spliceosome
Mouse model
Hematopoiesis
RNA-seq
U2AF1

3. OVERALL PROJECT SUMMARY:

Task 1. Seek IACUC and DoD ACURO approval for the use of animals.

<u>Current Objectives</u>: Obtain approval.
<u>Results</u>: IACUC and DoD ACURO approved.
<u>Progress and Accomplishments with Discussion</u>: Completed Task.

Task 2. Specific Aim 1. We will determine whether the U2AF1(S34F) mutation alters hematopoiesis in vivo.

<u>Current Objectives:</u> We will determine whether expression mutant U2AF1(S34F) induces ineffective hematopoiesis in mice. We will determine whether mutant U2AF1(S34F) contributes clonal dominance and MDS initiation.
<u>Results:</u> Figures from Task 2 results were reported in our year 1 update. Therefore, I will summarize these findings here without figures. U2AF1(S34F)-recipient mice have reduced total WBCs in the peripheral blood compared to U2AF1(WT)- and rtTA only-recipient controls (4.3 vs 7.11 and 7.13 K/μl, respectively, $p \leq 0.01$), but no significant changes in bone marrow cellularity or spleen size (n=9-11). U2AF1(S34F)-recipient mice have a perturbed mature cell lineage distribution, including reduced monocytes and B cells in both peripheral blood ($p \leq 0.05$)

and bone marrow ($p \leq 0.01$) when compared to control mice (n=9-11). Reduction of bone marrow monocytes occurs as early as 5 days and is associated with increased Annexin V+ ($p \leq 0.05$) and phospho-H2AX ($p \leq 0.05$) compared to controls, suggesting loss of these cells may be due to apoptosis. In addition, U2AF1(S34F)-recipient mice have increased numbers of progenitors in both bone marrow and spleen by CFU-C methylcellulose assay and flow cytometry for c-Kit+/Lineage- cells, as well as common myeloid progenitors (CMPs), when compared to U2AF1(WT) and rtTA only controls ($p \leq 0.05$, n=5-10). U2AF1(S34F)-recipient mice also have an increase in the frequency of bone marrow hematopoietic stem cells (HSCs) measured by flow cytometry for bone marrow KLS (c-Kit+/Lineage-/Sca-1+) cells ($p \leq 0.05$). The increase in bone marrow KLS cells in U2AF1(S34F)-recipient mice seen as early as 5 days is associated with higher levels of intracellular Ki67 (a marker of cell proliferation) in KLS cells compared to controls ($p<0.05$, n=8-13). Competitive repopulation studies show a disadvantage for bone marrow cells expressing mutant U2AF1(S34F) compared to U2AF1(WT) at ≥4 months post-transplant in both primary and secondary transplant recipient mice ($p \leq 0.05$, n=3-12), suggesting that the increase in KLS cell cycling following U2AF1(S34F) expression may lead to stem cell exhaustion.
Progress and Accomplishments with Discussion: Collectively, these data indicate U2AF1(S34F) expression alters hematopoiesis *in vivo*. These data have been published in Cancer Cell in May 2015.

Task 3. Specific Aim 2. We will use RNA-Seq to identify alternatively spliced genes in primary hematopoietic progenitor cells harvested from U2AF1(S34F) mutant mice.
Current Objectives: We will identify alternatively spliced genes in primary murine hematopoietic progenitor cells expressing mutant U2AF1 using RNA-Seq. We will validate alternatively spliced genes in primary MDS bone marrow cells expressing mutant U2AF1.
Results: We performed unbiased RNA sequencing on sorted bone marrow CMPs following 5 days of transgene induction in U2AF1(S34F)- and U2AF1(WT)-transplanted mice (n=3 each). Unsupervised clustering based on the splicing ratio of cassette and mutually exclusive exon junctions segregated U2AF1(S34F)-expressing CMP samples from U2AF1(WT) controls (**Figure 1A**). We identified 742 splicing junctions that were differentially expressed in 633 genes in U2AF1(S34F)/rtTA samples compared to U2AF1(WT)/rtTA controls (FDR<0.1). Using these 633 genes, we performed gene set enrichment and pathway analysis and identified an enrichment of genes involved in RNA binding (FDR<0.1) (**Figure 1B**). To further examine the effects of U2AF1(S34F) expression on splicing in vivo, we determined the consensus sequence flanking the AG dinucleotide at the 3' splice acceptor site known to be recognized by U2AF1 during splicing. In contrast to the sequence motif seen in non-dysregulated control junctions (**Figure 1C**), exons skipped more frequently by mutant U2AF1(S34F) relative to U2AF1(WT) were enriched for uracil (indicated by a T) in the -3 position relative to the AG dinucleotide (**Figure 1D**). We observed a similar pattern in alternative 3' splice site usage (i.e., junctions that did not involve exon skipping). Here, we saw an enrichment of T in the -3 position of canonical 3' splice sites whose alternative 3' splice site was utilized more frequently by mutant U2AF1(S34F) relative to U2AF1(WT) (**Figure 1E**).

To prioritize altered splicing events for further analysis, we intersected junctions that were significant (FDR<0.1) across 3 datasets: mouse CMP samples (n=219 junctions), AML patient samples with and without U2AF1 mutations (n=162 junctions), and our previously-described

primary human CD34+ cells over-expressing U2AF1(S34F) or U2AF1(WT) (n=1652 junctions). The intersection was (necessarily) composed of homologous junctions and was additionally restricted to include only concordantly dysregulated (same direction of log fold change) junctions. It included 17 homologous dysregulated junctions ($p<0.0001$; simulation) in 13 genes (**Figure 2**). Several of the dysregulated junctions corresponded to the same splicing event in a transcript and were identified as reciprocal splice junctions. All of these junctions occur in known isoforms, though the function of most of these isoforms remains largely unknown. Several of these junctions occurred in genes mutated in MDS and AML (GNAS, PICALM), or known to be involved in stem cell biology (H2AFY, MED24).

Next, we examined MDS patient bone marrow samples for mutant U2AF1-induced splicing changes discovered by RNA-seq analysis in a small subset of affected genes. Dysregulated splicing events were prioritized for validation by overlap in the 3 datasets, splicing ratio change, or known biological function of the gene, with particular focus on genes mutated in MDS. Mutant U2AF1-induced splice isoform changes identified by RNA-seq analysis were concordant with RT-PCR of MDS patient bone marrow samples for 7 of 8 splicing events examined, including H2AFY, MED24, GNAS, PICALM, KDM6A, KMT2D (MLL2) and BCOR; only EIF4A2 showed no difference (**Figure 3**).

<u>Progress and Accomplishments with Discussion:</u> Collectively, these data indicate U2AF1(S34F) expression alters pre-mRNA splicing *in vivo*. These splicing changes may contribute to disease pathogenesis in MDS. These data have been published in Cancer Cell in May 2015.

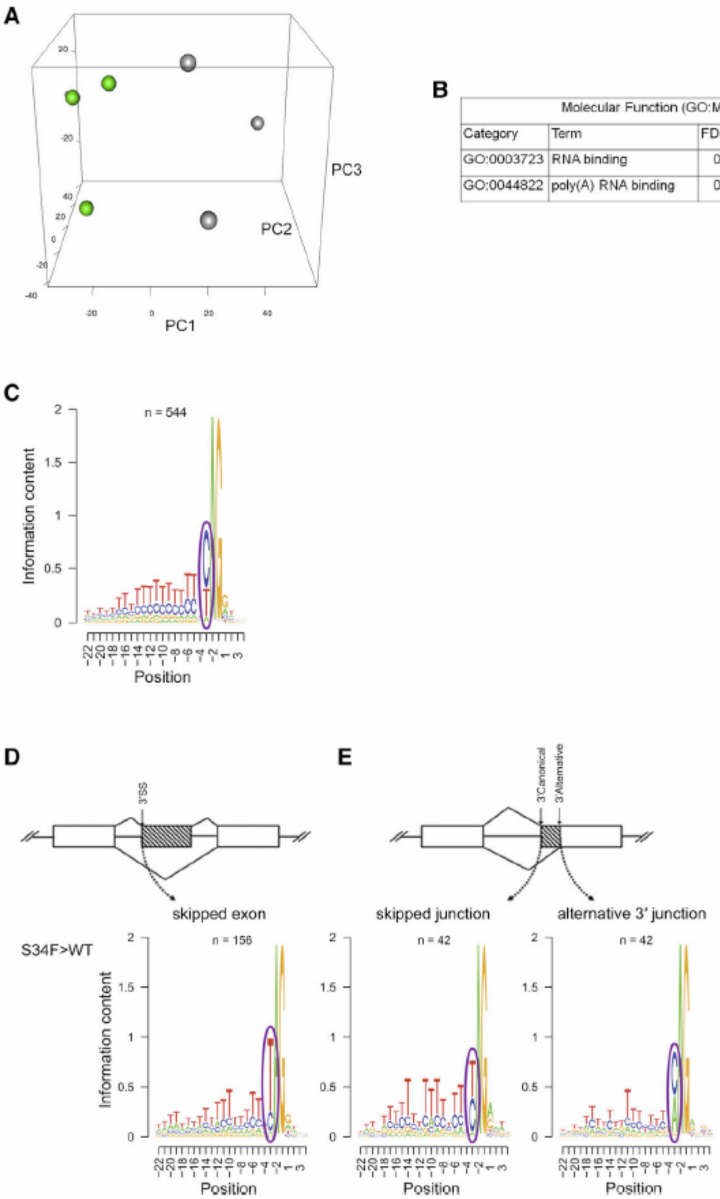

Figure 1. U2AF1(S34F) expression alters splicing in mouse progenitors cells *in vivo*. (A) Unsupervised principal component analysis of standardized splicing ratios of expressed 3' splice sites of cassette and mutually exclusive exons (> 5 reads in ≥ half of samples) in donor-derived bone marrow common myeloid progenitor cells (CMP) sorted from mice transplanted with U2AF1(S34F)/rtTA (green) or U2AF1(WT)/rtTA (grey) bone marrow (n=3 pools of 5-6 mice each) following 5 days doxycycline. (B) Pathways enriched (GOseq FDR<0.1) in differentially spliced genes in U2AF1 mutant CMPs. (C) Logos plot of the consensus sequences surrounding the AG dinucleotide of the 3' splice site of 544 randomly selected junctions not altered by U2AF1(S34F). (D) Logos plots of the 3' splice site consensus sequence in U2AF1(S34F)-skipped exons (n=156) and (E) skipped canonical splice junctions in favor of alternative 3' junctions (n=42) identified by DEXSeq (FDR<0.1). Results are depicted in Logos plots. ss, splice site.

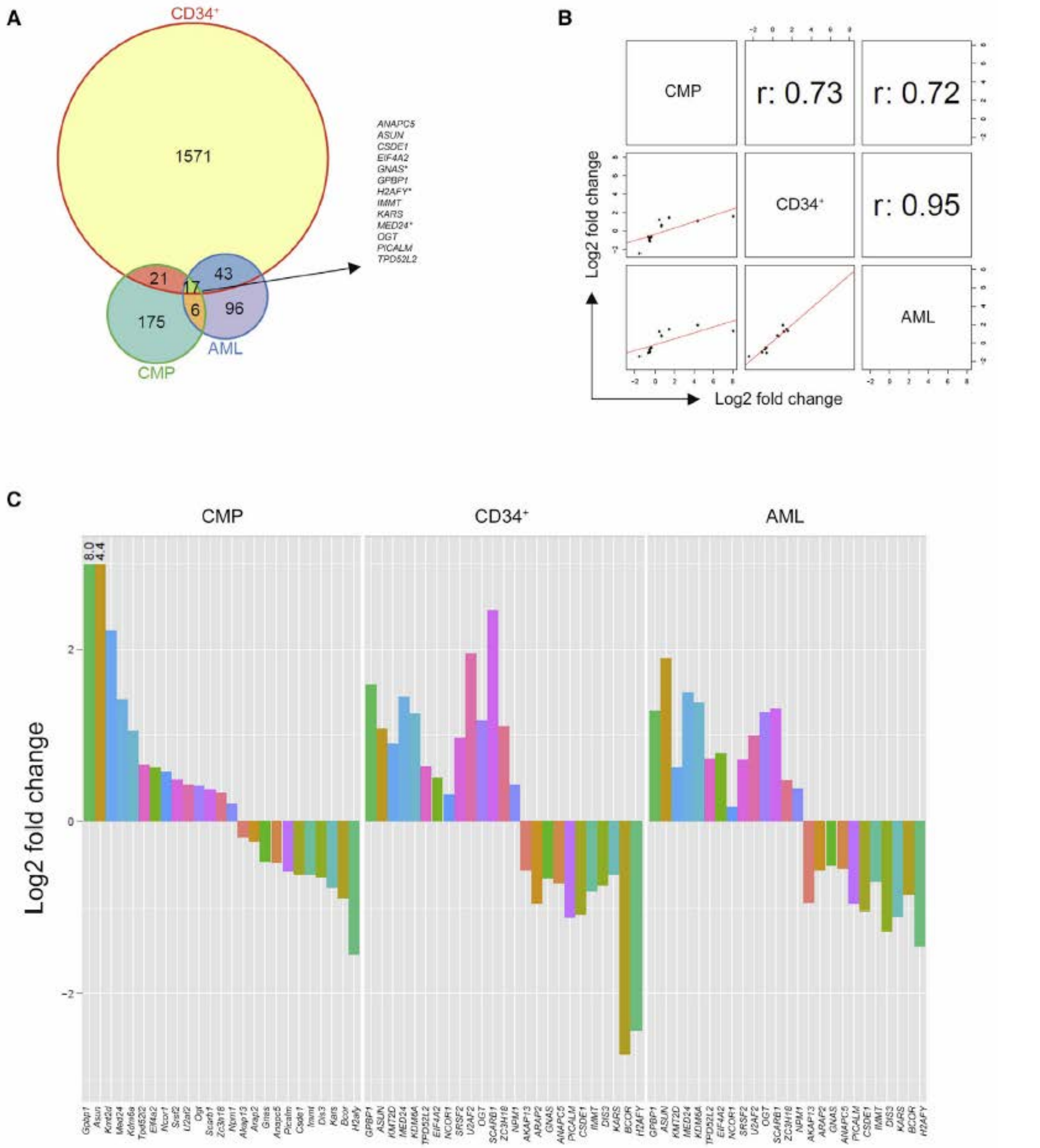

Figure 2. Mutant U2AF1 alters splicing in common targets across species. (A) Venn diagram displaying the overlap of independently discovered mutant U2AF1-induced splice junction changes by DEXSeq analysis (FDR<0.1) in 3 RNA-seq datasets: transgenic mouse CMPs, primary human hematopoietic CD34+ cells, and human AML patient samples. *Indicates multiple significant junctions detected within the gene. (B) Correlation plots and r values for junctions identified by overlap of DEXSeq datasets, as shown in the Venn diagram. Only one junction per gene is shown. (C) Log2 fold change in junction expression for overlapping genes in Figure 2A and enriched recurrently mutated genes in MDS and AML in U2AF1 mutant versus wildtype CMP cells, human CD34+ cells, and AML samples, plotted in descending order based on CMP results. *U2AF1* was excluded due to an inability to differentiate endogenous vs. exogenous U2AF1(WT) transcript in CD34+ cells.

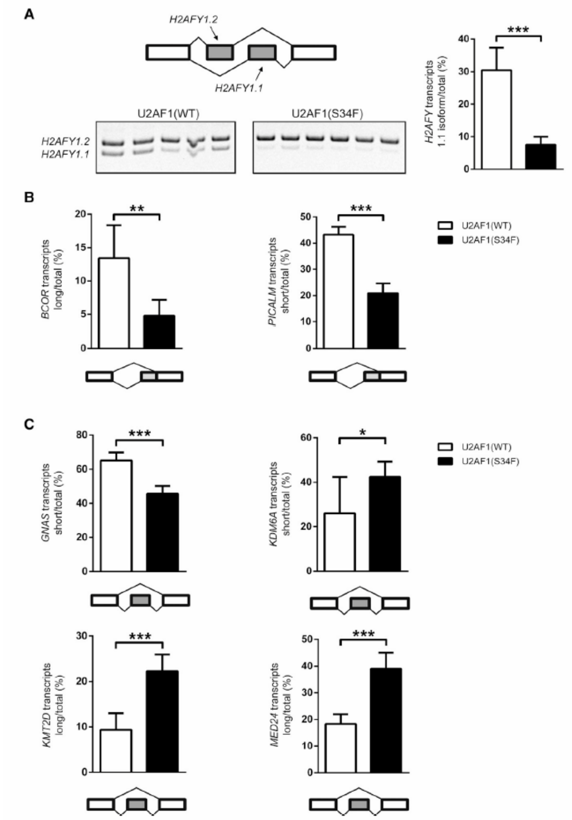

Figure 3. Mutant U2AF1 alters splicing in MDS bone marrow cells. (A) Mutually exclusive exons in H2AFY detected by RT-PCR and gel electrophoresis (quantified in right); the diagram of the event measured and the gel image (left). (B and C) Quantification of alternative splice site utilization (B) and altered cassette exon events (C). All data are represented as mean ±SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; $n = 5–6$.

Task 4. Data analysis and report generation
Current Objectives: Analyze data.
Results: Analysis is ongoing, as reported above. Analysis of hematopoietic subpopulations of cells for splicing alterations and comprehensive analysis of MDS bone marrow samples for splicing alterations is ongoing.
Progress and Accomplishments with Discussion: Anticipate completing analysis during the no cost extension year.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. Determined that mutant U2AF1 expression alters hematopoiesis *in vivo*.
2. Determined that mutant U2AF1 expression alters pre-mRNA splicing in primary mouse hematopoietic cells.
3. Identified several genes that are alternatively spliced in both U2AF1 mutant expressing mice and human hematopoietic cells, prioritizing these genes as candidates that may contribute to disease pathogenesis.

5. CONCLUSIONS:

The results provide evidence that spliceosome gene mutations, specifically *U2AF1* mutations, affect hematopoiesis and may contribute to bone marrow failure. Given that spliceosome gene mutations are the most common family of genes mutated in MDS, a better understanding of the underlying mechanisms of disease pathogenesis will have broad implications. Future studies will focus on long-term monitoring of mice for development of bone marrow failure or leukemia. We are investigating the mechanism underlying how specific gene isoforms that are induced by

mutant U2AF1 alter hematopoiesis. We will also identify additional splicing alterations induced by mutant U2AF1 in various stem, progenitor, and precursor hematopoietic cell populations that will be validated in primary MDS samples. Our long-term goal is to identify a core set of genes that are perturbed during differentiation that contribute to bone marrow failure.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Published Manuscripts:

1. Shirai CL, Ley JN, White BS, Kim S, Tibbitts J, Shao J, Ndonwi M, Wadugu B, Duncavage EJ, Okeyo-Owuor T, Liu T, Griffith M, McGrath S, Magrini V, Fulton RS, Fronick C, O'Laughlin M, Graubert TA, Walter MJ. Mutant U2AF1 Expression Alters Hematopoiesis and Pre-mRNA Splicing In Vivo. Cancer Cell. 2015 May 11;27(5):631-43. doi: 10.1016/j.ccell.2015.04.008. PubMed PMID: 25965570; PubMed Central PMCID: PMC4430854.

Abstracts:

1. Shirai CL, Ley JN, White B, Tibbitts J, Shao J, Ndonwi M, Kim S, Wadugu B, Okeyo-Owuor T, Graubert TA, Walter MJ. Mutant U2AF1 Expression Alters Hematopoiesis and Pre-mRNA Splicing In Transgenic Mice. American Society of Hematology, 56th Annual meeting, San Francisco, CA, USA. December, 2014.

Presentations:

1. Memorial Sloan-Kettering Cancer Center, Workshop on Splicing Factor Mutations in Cancer, New York, NY, USA. Title: Preclinical activity of a splicing modulator in U2AF1 mutant MDS/AML. October 27, 2015.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES: None.

11. APPENDICES: None.